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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **David Lewis, Hans Herweijer, James E. Hagstrom, Aaron Loomis, Jon A. Wolff,**	)	
Serial No.: **10/007,448**	)	Examiner: **Terra C. Gibbs**
Filed: **11/07/2001**	)	
Group Art Unit: **1635**	)	

For: **Inhibition Of Gene Expression By Delivery Of Small Interfering RNA To Post-Embryonic Animal Cells *In vivo***

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Examiner:

I, David Lewis, hereby declare as follows:

1. I am an inventor of the captioned application.

2. I submit with this Declaration and Response further experimental material (attached) illustrating: (a) inhibition of endogenous genes; cytosolic alanine aminotransferase, peroxisome proliferators-activated receptor, HMG Coenzyme A reductase, and green fluorescent protein following intravascular delivery of siRNA; (b) delivery of siRNA to heart, skeletal muscle, prostate and lung; (c) images demonstrating delivery of fluorescently labeled siRNA to liver; and (d) evidence that inhibition of gene expression by the delivered siRNA is not dependent on interaction of the siRNA with

a co-delivered plasmid prior to delivery. The experiments were performed according to the methods provided in the Specification.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

12/15/03

David Lewis Date

Inhibition of endogenous genes in the liver by delivery of siRNA

EXAMPLE 11 *(From Specification)*

Inhibition of endogenous mouse **cytosolic alanine aminotransferase** (ALT) expression after in vivo delivery of siRNA.

Single-stranded, cytosolic alanine aminotrasferase-specific sense and antisense RNA oligomers with overhanging 3' deoxynucleotides are prepared and purified by PAGE. The two oligomers, 40μM each, are annealed in 250μl of buffer containing 50mM Tris-HCl, pH 8.0 and 100mM NaCl, by heating to 94°C for 2 minutes, cooling to 90°C for 1 minute, then cooling to 20°C at a rate of 1°C per minute. The resulting siRNA is stored at -20°C prior to use.

The sense oligomer with identity to the endogenous mouse and rat gene encoding cytosolic alanine aminotransferase has the sequence: 5'-rCrArCrUrCrArGrUrCrUrCrUrArA-rGrGrGrCrUTT-3' and corresponds to positions 928-946 of the cytosolic alanine amino-transferase reading frame in the sense direction. The letter "r" preceding a nucleotide indicates that nucleotide is a ribonucleotide. The sense oligomer with identity to the endogenous mouse and rat gene encoding cytosolic alanine aminotransferase has the sequence: 5'-rArGrCrCrCrUrUrArGrArGrArCrUrGrArGrUrGTT-3' and corresponds to positions 928-946 of the cytosolic alanine aminotransferase reading frame in the antisense direction. The letter "r" preceding a nucleotide indicates that nucleotide is a ribonucleotide. The annealed oligomers containing cytosolic alanine aminotransferase coding sequence are referred to as siRNA-ALT

Mice are injected in the tail vein over 7-120 seconds with 40 μg of siRNA-ALT diluted in 1-3 mls Ringer's solution (147mM NaCl, 4mM KCl, 1.13mM $CaCl_2$). Control mice were injected with Ringer's solution without siRNA. Two days after injection, the livers were harvested and homogenized in 0.25 M sucrose. ALT activity was assayed using the Sigma diagnostics INFINITY ALT reagent according to the manufacturers instructions. Total protein was determined using the BioRad Protein Assay. **Mice injected with 40 μg of siRNA-ALT had a 32% average decrease in ALT specific activity compared to that of mice injected with Ringer's solution alone.** (bold added)

Inhibition of PPAR expression in mice by delivery of **PPAR**-specific siRNA.

PPARα, peroxisome proliferator-activated receptor α, is a transcription factor and member of the nuclear hormone receptor superfamily. This gene, found in both mice and humans, plays an important role in the regulation of mammalian metabolism. In particular, PPARα is required for the normal maintenance of metabolic pathways whose misregulation can facilitate the development of hyperlipidemia and diabetes. When bound to its ligand, PPARα binds to the retinoid X receptor (RXR) and activates the transcription of genes implicated in maintaining homeostatic levels of serum lipids and glucose. The manipulation of PPARα levels using RNA interference may be a safe and effective way to modulate mammalian metabolism and treat pathogenic hyperlipidemia and diabetes. We used the tail vein injection procedure to modulate endogenous PPARα levels using RNA interference in mice. Our results provide a model for the therapeutic delivery of siRNA. This method, or variations thereof, will be useful in the modulation of the levels of an endogenous gene using RNA interference.

Initially, we identified a series of siRNAs that exhibited RNAi activity against PPARα in primary cultured hepatocytes. Having identified several highly active siRNAs, we selected one to use in our *in vivo* demonstration of siRNA delivery.

siRNA sequences. All RNA sequences were ordered from Dharmacon, Inc. The siRNA duplex directed against PPARα contained the target sequence 5'-rGrArTrCrGrGrAr-GrCrTrG-rCrArArGrArTrTrC-3'. A control GL3 siRNA duplex contained the target sequence 5'rArArCrUrUrArCrGrCrUrGrArGrUrArCrUrUrCrGrA-3'. The "r" between each indicated base is used to indicate that the oligonucleotides are ribooligonucleotides. All siRNAs contained dTdT overhangs.

Injections of mice. Four mice in each experimental group were injected with 50 μg of siRNA using the tail vein procedure. A volume of Ringer's solution (147 mM NaCl, 4 mM KCl, 1.13 mM $CaCl_2$) corresponding to 10% of each animal's body weight and containing 50 μg of PPARα siRNA sequences (or controls) were injected into mice over a period of 10 seconds. After 48 hours, livers from injected mice were harvested and total RNA was isolated.

Isolation of total RNA and cDNA synthesis. Total mRNA from injected mouse livers was isolated using Tri-Reagent. 500 ng of ethanol precipitated, total RNA suspended in RNase-free water was used to synthesize the first strand cDNA using SuperScript III reverse transcriptase. cDNAs were then diluted 1:50 and analyzed by quantitative, real-time qPCR.

Quantitative, real-time PCR. Bio-Rad's iCycler quantitative qPCR system was used to analyze the amplification of PPARα and GAPDH amplicons in real time. The intercalating agent SYBR Green was used to monitor the levels of the amplicons. Primer sequences used to amplify PPARα sequences were 5'-TCGGGATGTCACACAATGC-3' and 5'-AGGCTTCGTGGATTCTCTTG-3'. Primer sequences used to amplify GAPDH sequences were 5'-CCTCTATATCCGTTTCCAGTC-3' and 5'-TTGTCGGTGCAAT-AGTTCC-3'. Primers used to amplify PTEN sequences were 5'-GGGAAGTAAGGAC-CAGAGAC-3' and 5'-ATCATCTTGTGAAACAGCAGTG-3'. Serial dilutions (1:20, 1:100 and 1:500) of cDNA made from Ringer's control samples were used to create the standard curve from which mRNA levels were determined.

RESULTS- Mouse livers injected with siRNAs directed against PPARα contained 17% or 37% less PPARα mRNA than Ringer's control or GL3 siRNA control animals, respectively. Fig. 1 shows the relative levels of PPARα mRNA as compared to total input RNA in each four-mouse group. The experimental error is expressed as the standard deviation of the mean. Fig. 2 shows the relative levels of PPARα mRNA in each individual mouse of the three experimental groups. The experimental error in Fig. 2 is expressed as the standard deviation of each three-replicate measurement.

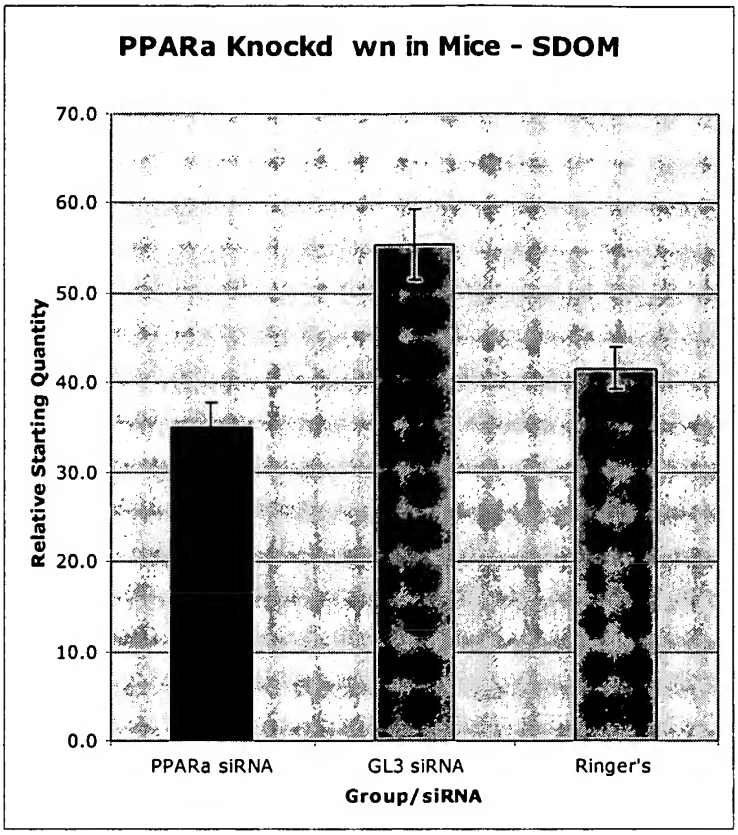


Figure 1. Relative levels of PPARα mRNA in groups of mice injected with siRNAs. mRNA levels are shown relative to total input RNA. Black bar = Experimental group; Grey bars = control groups.

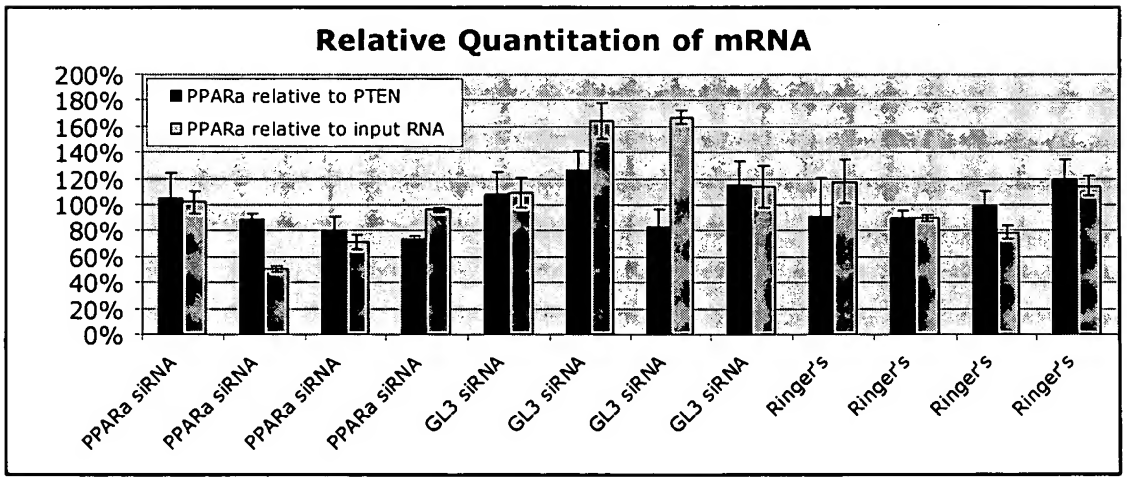


Figure 2. Relative levels of PPARα mRNA in individual mice injected with siRNAs. mRNA levels are shown relative to both total input RNA (grey bars) and PTEN mRNA (black bars).

Inhibition of **PPAR** expression in mice by delivery of PPAR-specific siRNA expression cassettes. Gene silencing can also be initiated in mammalian cells by transfection with an expression vector producing the siRNA using the cells' own transcription machinery. We describe here the effective delivery of siRNAs via their synthesis from delivered plasmid DNA. The activity of siRNAs delivered to cells in vivo exerts an effect on the levels of a therapeutically important enzyme.

Initially, we identified a series of plasmid DNA-based siRNA hairpins that exhibited RNAi activity against PPARα in primary cultured hepatocytes. Having identified several active siRNA hairpins, we selected one to use in our *in vivo* demonstration of siRNA delivery.

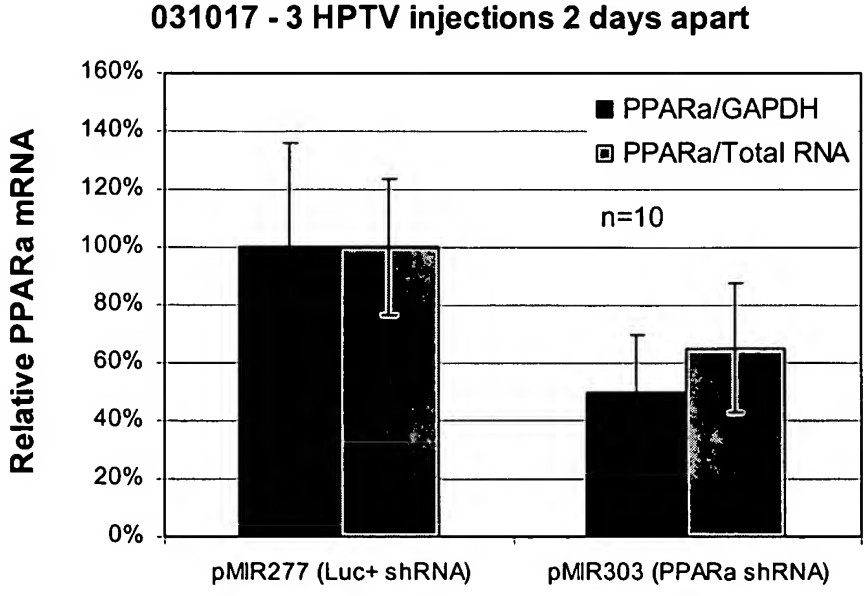
siRNA hairpin sequences. The general hairpin structure consists of a single polynucleotide sequence with sense and antisense target sequences flanking a micro-RNA hairpin loop structure. Transcription of the siRNA hairpin constructs was driven by the promoter from the human U6 gene. In addition, the end of the hairpin construct contains five T's to serve as an RNA Polymerase III termination sequence. The siRNA hairpin directed against PPARα had the sequence 5′-GGAGCTTTGGGAAGAGGAAGGTGTCATCcttcctgtcaGATGGCATCT-TCCTCTTCCCGAAGCTCCTTTTT-3′. Lower-case letters indicate the sequence of the hairpin loop motif. The entire hairpin construct encoding the PPARα siRNA (consisting of the U6 promoter, the PPARα siRNA hairpin, and the termination sequence) is referred to as pMIR303. The negative control siRNA hairpin directed against GL3 had the sequence 5′-GGATTCCAATTCAGCGGGAGCCACCTGATgaagcttgATCGGGTGGCTCTCGCTG-AGTTGGAATCCATTTTT-3′. The entire hairpin construct encoding the GL3 siRNA (consisting of the U6 promoter, the GL3 siRNA hairpin, and the termination sequence) is referred to as pMIR277.

Injections of mice. Ten mice in each experimental group were injected three times each with 40 μg/injection of either pMIR277 (GL3 siRNA construct) or pMIR303 (PPARα siRNA construct) using a tail vein injection procedure. Volumes of Ringer's solution (147 mM NaCl, 4 mM KCl, 1.13 mM $CaCl_2$) corresponding to 10% of each animal's body weight and containing the 40 μg of pMIR277 or pMIR303 were injected into mice over a period of 10 seconds with each injection. For each animal, injection 1 was performed on Day 0, injection 2 was performed on Day 2, and injection 3 was performed on Day 4. Seven days after Injection 3 (Day 11), livers from all mice were harvested and total RNA was isolated using the Tri-Reagent protocol.

Isolation of total RNA and cDNA synthesis. Total mRNA from injected mouse livers was isolated using Tri-Reagent. 500 ng of ethanol precipitated, total RNA suspended in RNase-free water was used to synthesize the first strand cDNA using SuperScript III reverse transcriptase. cDNAs were then diluted 1:50 and analyzed by quantitative, real-time qPCR.

Quantitative, real-time PCR. Bio-Rad's iCycler quantitative qPCR system was used to analyze the amplification of PPARα and GAPDH amplicons in real time. The intercalating agent SYBR Green was used to monitor the levels of the amplicons. Primer sequences used to amplify PPARα sequences were 5′-TCGGGATGTCACACAATGC-3′ and 5′-AGGCTTCGTGGATTCTCTTG-3′. Primer sequences used to amplify GAPDH sequences were 5′-CCTCTATATCCGTTTCCAGTC-3′ and 5′-TTGTCG-GTGCAATAGTTCC-3′. Serial dilutions (1:20, 1:100 and 1:500) of cDNA made from Ringer's control samples were used to create the standard curve from which mRNA levels were determined. PPARα levels were quantitated relative to both GAPDH mRNA and total input RNA.

RESULTS- Mouse livers injected with the PPARα hairpin constructs contained 50% or 35% less PPARα mRNA than those injected with GL3 siRNA control hairpins when compared to GAPDH mRNA or total input RNA, respectively. Fig. 1 shows the relative levels of PPARα mRNA as compared to GAPDH mRNA or total input RNA in each 10-mouse group. The experimental error is expressed as the total standard deviation among all samples. That this delivery procedure is able to achieve up to 50% knockdown of an endogenous target transcript demonstrates its general utility for *in vivo* modulation of gene expression.



Reduction of serum triglyceride levels using siRNA to inhibit **HMG CoA reductase** *in vivo.*

We have demonstrated a reduction of serum triglyceride levels in mice upon treatment with siRNA directed against HMG CoA reductase. Group A (series2) mice (5 mice) were each injected with 50 μg of an siRNA directed against mouse HMG CoA reductase mRNA. Group B (Series1) mice (5 mice) were an uninjected control group. Group A and Group B animals were bled 7 days before, 2 days after, 4 days after, and 7 days after the injection. Serum samples were stored at −20°C until all timepoints had been collected. Each group's serum samples from a given time-point were pooled prior to the triglyceride assays. Triglyceride assays were performed in quintuplicate.

Mice. Experiments were performed in *Apoetm1Unc* mice obtained from The Jackson Laboratories (Bar Harbor, ME). Mice homozygous for the *Apoetm1Unc* mutation show a marked increase in total plasma cholesterol levels that is unaffected by age or sex. Moderately increased triglyceride levels have been reported in mice with this mutation on a mixed C57BL/6 x 129 genetic background.

siRNA reagents. Single-stranded, HMG CoA reductase-specific sense and antisense RNA oligomers with overhanging 3' deoxyribonucleotides were ordered from Dharmacon, Inc. The annealed RNA duplex was resuspended in Buffer A (20 mM KCl, 6 mM HEPES-KOH pH 7.5, 0.2 mM $MgCl_2$) and stored at −20°C prior to use. Prior to injection, siRNAs were diluted to the desired concentration (50 μg/2.2 ml) in Ringer's solution.

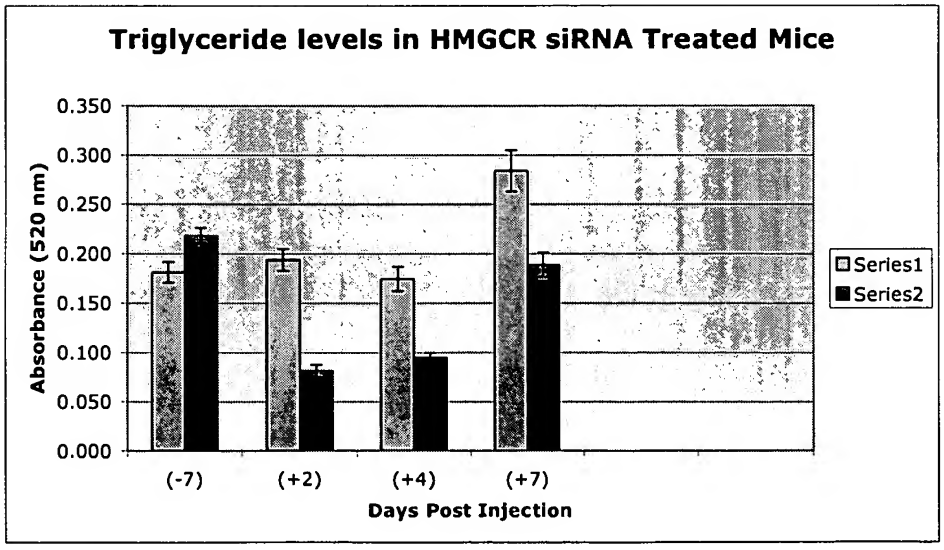
Oligonucleotide sequences. The sense oligomer with identity to the murine HMG CoA reductase gene has the sequence: SEQ ID NO. 1: 5'-rArCrArUrUrGrUrCrArCrUrGrC-rUrArUrCrUrATT-3', which corresponds to positions 2324-2344 of the HMG CoA reductase reading frame in the sense direction. The letter "r" preceding each nucleotide indicates that nucleotide is a ribonucleotide. The antisense oligomer with identity to the murine HMG CoA reductase gene has the sequence: SEQ ID NO. 2: 5'-rUrArGrArUrArGrCrArGrUrGrArC-rArArUrGrUTT-3', which corresponds to positions 2324-2344 of the HMG CoA reductase reading frame in the antisense direction. The letter "r" preceding each nucleotide indicates that nucleotide is a ribonucleotide. The annealed oligomers containing HMG CoA reductase coding sequence are referred to as siRNA-HMGCR.

A total of 50 μg of siRNA-HMGCR was dissolved in 2.2 ml Ringer's solution (147mM NaCl, 4mM KCl, 1.13mM $CaCl_2$), and injected into the tail vein of ApoE (-/-) mice over 7-12 seconds. Control mice were not injected and are referred to here as naive. Each mouse was bled from the retro-orbital sinus at various times prior to and after injection. Cells and clotting factors were pelleted from the blood to obtain serum. The serum triglyceride levels

were then assayed by a enzymatic, colorometric assay using the Infinity Triglyceride Reagent (Sigma Co.). Results showed that triglyceride levels in siRNA-HMGCR treated mice (Series2) were reduced 62% after two days, 56% after two days, and returned to normal levels after 7 days. No decrease in serum triglyceride levels was observed in uninjected mice (Series1).

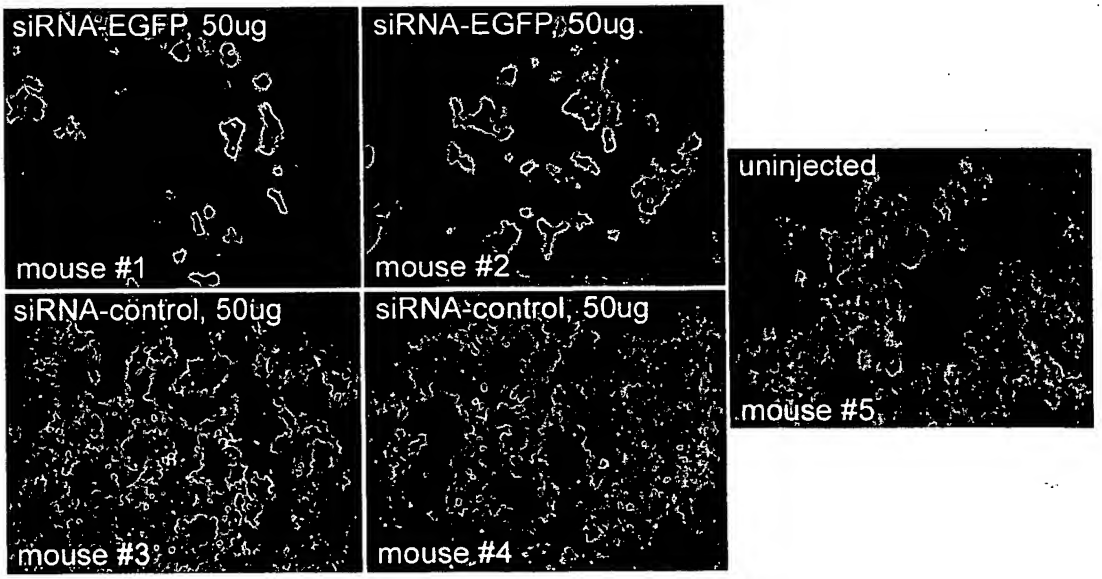
Triglyceride assays. Serum samples were diluted 1:100 in the Infinity Triglyceride Reagent (2 µl in 200 µl) in a clear, 96-well plate. Each assay plate was then incubated at 37°C for five minutes, removed and allowed to cool to room temperature. Absorbance was measured at 520 nm using a SpectraMax Plus plate reader (Molecular Devices, Inc). Background absorbance (no serum added) was subtracted from each reading and the resulted data was plotted versus timepoint.

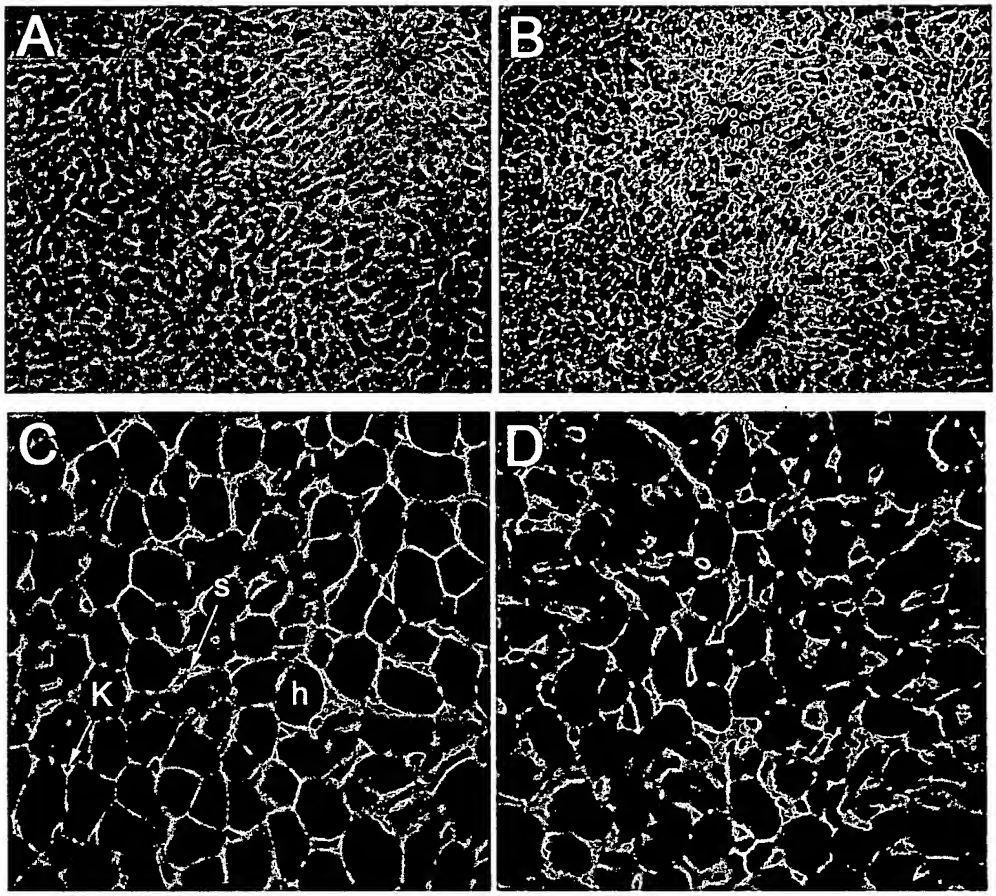
	Triglyceride levels			
	−7 days	+2 days	+4 days	+7 days
No injection control (n = 4)				
average	0.181	0.194	0.175	0.284
st. dev.	0.010	0.011	0.012	0.021
siRNA-HMGCR Treated animals (n = 5)				
average	0.218	0.082	0.095	0.189
st. dev.	0.008	0.006	0.005	0.012



series 1 = no injection control; series 2 = siRNA-HMGCR treatment

Inhibition of **green fluorescent protein** in transgenic mice using siRNA. The commercially available mouse strain C57BL/6-TgN(ACTbEGFP)10sb (The Jackson Laboratory) has been reported to express enhanced green fluorescent protein (EGFP) in all cell types except erythrocytes and hair. These mice were injected with siRNA targeted against EGFP (siRNA-EGFP) or a control siRNA (siRNA-control) using the increased pressure tail vein intravascular injection method described previously. 30 h post-injection, the animals were sacrificed and sections of the liver were prepared for fluorescence microscopy. Liver sections from animals injected with 50 μg siRNA-EGFP displayed a substantial decrease in the number of cells expressing EGFP compared to animals injected with siRNA-control or mock injected. The data shown here demonstrate effective delivery of siRNA-EGFP to the liver. The delivered siRNA-EGFP then inhibited EGFP gene expression in the mice. We have therefore shown the ability of siRNA to inhibit the expression of an endogenous gene product in post-natal mammals.





Inhibition of co-delivered genes in the various tissues by intravascular deliver of siRNA

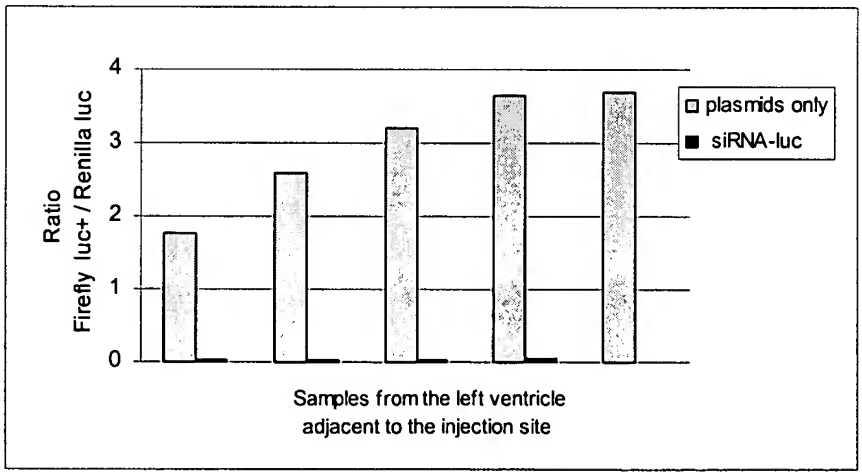
Inhibition of Luciferase expression in **pig heart** by delivery of siRNA

Pig #1 – This animal was injected with plasmids only. The injection solution was prepared by adding 100 μg/ml each of Fireflyluc+ and Renillaluc to a saline solution which also contained 2.5 mg/ml of lidocaine. The injection volume for this animal was 12.5 ml and the rate of injection was 4.5 ml/second.

Pig #2 – This animal was injected with plasmids and the siRNA-luc+. The injection solution was prepared by adding 100 μg/ml each of Fireflyluc+ and Renillaluc and 45μg/ml of siRNA-luc+. The injection solution was saline with 2.5 mg/ml of lidocaine. The injection volume for this animal was 20 ml and the rate was 5.0 ml/second.

The animals was sacrificed at 48 hours and the heart was excised. Tissue specimens (approximately 1 gram each) were obtained near the injection site from the muscle surrounding the left anterior descending artery and vein. Specimens were frozen in liquid N2 and stored at −80°C. Expression levels were measured by preparing homogenates and measuring activity of the firefly luciferase+ and the renilla luciferase using a commercial available assay kit (Promega). Data is expressed as a ratio fireflyluc+ / renillaluc.

The data show that siRNA was efficiently to heart muscle cells near the left anterior descending artery and vein vasculature following retrograde injection of a solution containing the siRNA into the left anterior descending vein. In animals receiving siRNA, firefly luciferase expression was reduced 70-90% (ratio of firefly luciferase to *Renilla* luciferase in control vs. siRNA animals).



Inhibition of Luciferase expression in **muscle** by delivery of siRNA into the iliac artery.

A. *Iliac injection* – (from example 9 of the specification)10 μg pGL3 control and 1 μg pRL-SV40 with 5.0 μg siRNA-luc+ or 5.0 siRNA-ori were injected into iliac artery of rats. Specifically, animals were anesthetized and the surgical field shaved and prepped with an antiseptic. The animals were placed on a heating pad to prevent loss of body heat during the surgical procedure. A midline abdominal incision was made after which skin flaps were folded away and held with clamps to expose the target area. A moist gauze was applied to prevent excessive drying of internal organs. Intestines were moved to visualize the iliac veins and arteries. Microvessel clips were placed on the external iliac, caudal epigastric, internal iliac, deferent duct, and gluteal arteries and veins to block both outflow and inflow of the blood to the leg. An efflux enhancer solution (e.g., 0.5 mg papaverine in 3 ml saline) was injected into the external iliac artery though a 25 g needle, followed by the plasmid DNA and siRNA containing solution (in 10 ml saline) 1-10 minutes later. The solution was injected in approximately 10 seconds. The microvessel clips were removed 2 minutes after the injection and bleeding was controlled with pressure and gel foam. The abdominal muscles and skin were closed with 4-0 dexon suture.

Four days after injection, rats were sacrificed and the quadriceps and gastrocnemius muscles were harvested and homogenized. Luc+ and Renilla Luc activities were assayed using the Dual Luciferase Reporter Assay System (Promega). Ratios of Luc+ to Renilla Luc were normalized to the siRNA-ori control. siRNA-Luc+ inhibited Luc+ expression in quadriceps and gastrocnemius by 85% and 92%, respectively, compared to the control siRNA-ori. Thus siRNA was effectively delivered to muscle cells in the leg using the delivery procedure.

Intravenous injection provides effective delivery of polynucleotide to limb skeletal muscle.

120-140 g adult Sprague-Dawley rats were anesthetized with 80 mg/kg ketamine and 40 mg/kg xylazine and the surgical field was shaved and prepped with an antiseptic. The animals were placed on a heating pad to prevent loss of body heat during the surgical procedure. A 4 cm long abdominal midline incision was made after which skin flaps were folded away and held with clamps to expose the target area. A moist gauze was applied to prevent excessive drying of internal organs. Intestines were moved to visualize the iliac veins and arteries. Microvessel clips were placed on the external iliac, caudal epigastric, internal iliac, deferent duct, and gluteal arteries and veins as well as on the inferior vena cava near the bifurcation to block both outflow and inflow of the blood to the leg. An efflux enhancer solution (e.g., 0.5 mg papaverine in 3 ml saline) was injected into the external iliac artery though a 25 g needle. 1-10 minutes later, a 27 G butterfly needle was inserted into the lateral saphenous vein and 10.5 ml normal saline containing 500 μg pMIR48 plasmid DNA encoding firefly Luciferase was injected at a rate of 0.583 ml/sec. Fluid was injected in the direction of normal blood flow. The microvessel clips were removed 2 minutes after the injection and bleeding was controlled with pressure and gel foam. The abdominal muscles and skin were closed with 4-0 dexon suture.

Luciferase activity (relative light units)							
animal	Quad	Biceps	Hamstring	Gastroc	Shin	Foot	total
1	18510700	5633280	2421540	4058470	8732620	5240	39361850
2	44406070	20210730	15864580	3019110	27226910	27210	110754610
3	10689250	5126590	666520	1860300	1041520	4650	19388830

Delivery of plasmid DNA and siRNA to the **prostate** via the bladder. ICR mice were anesthetized with 1-2% isoflurane and a midline incision was made extending from the pubis to the mid-abdomen. Retractors were positioned to expose the bladder and male reproductive organs. Curved vascular clamps were placed on the base of the bladder and the base of the seminal vesicles. Micro vascular clamps were also placed on the right and left ureters, the right and left deferent ducts and the urethral opening. A 30-gauge blunt needle catheter was inserted into the base of the bladder below the vascular clamp. The catheter was connected to a syringe pump and 50 μg pMIR48 + 4 μg pMIR122 plasmid DNA + 25 μg GL3-153 siRNA or 25 μg EGFP-64 siRNA in 0.5 ml Ringer's was injected at a rate of 3 ml/min. Two min after the injection the catheter was removed and the needle hole sealed with a hemostatic sponge. All clamps were removed and the abdominal wall was closed in two layers with 4-0 suture. The results show efficient delivery of plasmid DNA and siRNA to the prostate. Delivery of the plasmid results in expression of genes encoded on the plasmid. Delivery of the siRNA results in specific inhibition of gene expression.

Table 5. Intravascular delivery of plasmid DNA and siRNA to prostate

siRNA	Luciferase Activity		
	firefly	*Renilla*	firefly/ *Renilla*
25μg GL3-153	4330	16190	0.27
	120720	287590	0.42
	34120	41320	0.83
	2730	7890	0.35
		mean	0.46
25μg EGFP-64	239070	22310	10.72
	43760	9490	4.61
	561210	49280	11.39
	371810	52830	7.04
		mean	8.44

Delivery of polynucleotides to the **prostate** by injection into the penis vein or bladder.

A. Injection into bladder: ICR mice were anesthetized with 1-2% isoflurane and a midline incision was made extending from the pubis to the mid-abdomen. Retractors were positioned to expose the bladder and male reproductive organs. Curved vascular clamps were placed on the base of the bladder and the base of the seminal vesicles. Micro vascular clamps were also placed on the right and left ureters, the right and left deferent ducts and the urethral opening. A 30-gauge blunt needle catheter was inserted into the base of the bladder below the vascular clamp. The catheter was connected to a syringe pump and 20 μg pMIR48 plasmid DNA in 500 μl ringers was injected at a rate of 3 ml/min. Two min after injection the catheter was removed and the needle hole sealed with a hemostatic sponge. All clamps were removed and the abdominal wall was closed in two layers with 4-0 suture. Alternatively, the solution was injected directly into the prostate, 500 μl at 3 ml/min. The direct injection was performed using the same procedure as the bladder injections, including clamps, except that the solution was injected directly into the prostate. 24 h after injection, organs were harvested in the indicated volume of lysis buffer and 10 μl was assayed for luciferase activity.

B. Injection into tail vein: Tail vein injections of ~1.0 mL per 10 g body weight were preformed on ICR mice (n = 2) using a 30 gauge, 0.5 inch needle. The injection solution contained 20 μg pMIR48 plasmid DNA in Ringer's. Injections were done manually with injection times of 4-5 sec [Zhang et al. 1999; Liu et al. 1999]. One day after injection, the prostates were harvested and homogenized in lysis buffer (0.1% Triton X-100, 0.1 M K-phosphate, 1 mM DTT, pH 7.8). Insoluble material was cleared by centrifugation and 10 μl of the cellular extract or was analyzed for luciferase activity as previously reported [Wolff et al 1990].

C. Injection into penis vein: ICR mice were anesthetized with 1-2% isoflurane and a midline incision was made extending from the pubis to the mid-abdomen. Retractors were positioned to expose the bladder and male reproductive organs. 20 μg pMIR48 plasmid DNA in 2.3 ml Ringer's was injected by hand into the dorsal vein of the penis at a rate of 3 ml/min. Because no clamps were used in this experiment, and because of the presence of connecting veins, this injection is similar to injection into tail vein. 24 h after injection, organs were harvested in the indicated volume of lysis buffer and 10 μl was assayed for luciferase activity.

D. Injection of DNA complexes: Plasmid DNA was combined with histone and the lipid MC798 at a weight ratio of 1:3:1 to form cationic transfection complexes in a low salt isotonic glucose solution.

Animals were anesthetized with 1-2% isoflurane and a midline incision was made extending from the pubis to the mid-abdomen. Retractors were positioned to expose the bladder and male reproductive organs. Microvessel clamps were applied to the inferior vena cava and the anastomotic veins of the prostate just prior to injecting the polynucleotide. 200 µl of the polynucleotide-containing solution was injected into the dorsal vein of the penis in <7 sec. Clamps were removed 5-10 sec after the injection. Mice were sacrificed 24 hours after injection and various organs were assayed for luciferase expression. Luciferase activity is expressed as total relative light units per organ. The procedure resulted in high level reporter gene expression in the prostate.

Table 2. Delivery of polynucleotides to prostate

vol injected (ml)	lysis vol (ml)	animal	RLUs
A. Injection into bladder with clamps			
0.5	0.5	1	12,285
		2	1,144,862
		3	79,390
		average	412,179
B. Injection into tail vein			
2.3	0.5	1	1,435
C. Injection into penis vein			
2.3	0.5	1	457,949
		2	201,597
		average	329,773
D. H1 lipid derivative w/isotonic glucose - direct injection			
0.2	0.5	1	412,879
E. H1 lipid derivative w/isotonic glucose - penis vein			
0.2	0.5	1	264,479
		2	236,785
		average	250,632

Inhibition of Luciferase expression in **lung** after in vivo delivery of siRNA using recharged particles. Recharged particles were formed to deliver the reporter genes luciferase+ and Renilla luc as well as siRNA targeted against luciferase+ mRNA or a control siRNA to the lung. In this experiment, particles containing the reporter genes were delivered first, followed by delivery of particles containing the siRNAs. In all cases, particles were prepared with the polycation linear polyethylenimine (lPEI)and the polyanion polyacrylic acid (pAA). For delivery of reporter genes, particles were prepared which contained a mixture of the luc+ and Renilla luc expression plasmids. Normalization of expression of the two luciferase genes corrects for varying plasmid delivery efficiencies between animals. Particles containing a mixture of the expression plasmids containing the luciferase+ gene and the Renilla luciferase gene were injected intravascularly. Particles containing siRNA-Luc+ or a control siRNA were injected intravascularly immediately following injection of the plasmid-containing particles. 24 hours later, the lungs were harvested and the homogenate assayed for both Luc+ and Renilla Luc activity.

Specific experimental details were as follows: plasmid-containing particles were prepared by mixing 45 μg pGL3 control (Luc+) and 5 μg pRL-SV40 (Renilla Luc) with 300 μg lPEI in 10 mM HEPES, pH 7.5/5% glucose. After vortexing for 30 seconds, 50 μg pAA was added and the solution vortexed was for 30 seconds. siRNA-containing particles were prepared similarly, except 25 μg siRNA was used with 200 μg lPEI and 25 μg pAA. Particles containing the plasmid DNAs (total volume 250 μl) were injected into the tail vein of ICR mice. In animals that received siRNA, particles containing siRNA (total volume 100 μl) were injected into the tail vein immediately after injection of the plasmid DNA-containing particles. 1.5 mg pAA in 100 μl was then injected into the tail vein some animal 0.5 h later. 24 h later, animals were sacrificed and the lungs were harvested and homogenized. The homogenate was assayed for Luc+ and Renilla Luc activity using the Dual Luciferase Assay Kit (Promega Corporation).

Results indicate that intravascular injection of particles containing the plasmids pGL3 control and pRL-SV40 results in Luc+ and Renilla Luc expression in lung tissue (Table 2). Injection of particles containing siRNA-Luc+ after injection of the plasmid-containing particles resulted in specific inhibition of Luc+ expression. Renilla Luc expression was not inhibited. Injection of particles containing control siRNA (siRNA-c), targeted against an unrelated gene product did not result in inhibition of either Luc+ or Renilla Luc activity, demonstrating that the effect of siRNA-Luc+ on Luc+ expression is sequence specific and that injection of siRNA particles per se does not generally inhibit delivery or expression of

delivered plasmid genes. These results demonstrate that particles formed with lPEI and pAA containing siRNA are able to deliver siRNA to the lung and that the siRNA cargo is biologically active once inside lung cells.

Table 5. Delivery of siRNA to the lung using recharged particles results in inhibition of target gene expression.

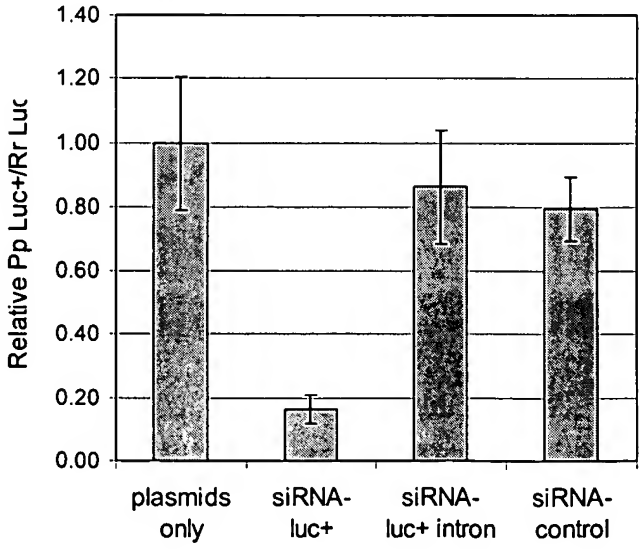
Particles	Relative light units		Average Luc+/ Renilla Luc ratio	Normalized Luc+/Renilla Luc
	Replicate 1	Replicate 2		
plasmids only				
Luc+	560994	680038	0.43 +/- 0.05	1.00
Renilla Luc	1406188	1452593		
siRNA-Luc+				
Luc+	326697	428079	0.21 +/- 0.07	0.48 +/- 0.16
Renilla Luc	1283313	2683842		
siRNA-c				
Luc+	964503	1452962	0.37 +/- 0.01	0.86 +/- 0.03
Renilla Luc	2527933	4005381		

Images: Delivery of fluorescently labeled siRNA to liver hepatocytes, low volume vs. increased permeability injection.

Injection of siRNA using increased pressure results in siRNA uptake in liver cells. Mice were injected into the tail vein with 35μg of siRNA labeled with Label-IT Cy3. Livers were harvested 30 minutes after injection and whole sections fixed in 4% parafomaldehyde, The fixed liver tissue was then soaked in 30 % sucrose and frozen in OCT medium for preparation of thin sections. Thin sections were counterstained with Alexa-488 phalloidin (Molecular Probes) diluted 1:400 in PBS. The sections were rinsed in PBS and then overlayed with VectaShield mounting medium (Vector Laboratories) prior to microscopy. Panels A and B are low magnification images of liver sections captured with a Zeis AxioCam using a 20x objective lens on a Zeis Axioplan 2 microscope. Panels C and D are high magnification images of liver sections captured on a Zeis LSM confocal microscope using a 63x objective lens. Panels A and C, liver sections from mice injected with Cy3-labeled siRNA under normal conditions (100μl Ringer's, 5-7 seconds). Panels B and D, liver sections from mice injected with Cy3-labeled siRNA under increased pressure (2 ml Ringer's solution in 5-7 seconds). Different cells and structures in the liver sections are indicated in Panel C. h = a representative hepatocyte; s = a representative sinusoid; K = a representative Kupffer cell. Note that in panels A and C, fluorescent label in observed in Kupffer cells, but little to no siRNA is present in hepatocytes. In contrast, in panels B and D, high levels of siRNA are observed in a majority of the hepatocytes.

siRNA does not inhibit expression of a co-delivered plasmid-encoded target gene through interaction prior to delivery to the cell.

Co-delivery of target plasmid with siRNA with sequence identical to a target gene intron does not result in target gene knockdown in mouse liver. Mice (n=4) were injected in the tail vein with a solution containing plasmids pMIR150 (0.5μg), expressing Pp Luc+, and pMIR252 (40μg), expressing Rr Luc with or without siRNAs. The volume injected was 1 ml per 10 g body weight at a rate of 5-20 seconds per injection. The siRNAs targeted the coding region of Pp Luc+ (siRNA-luc+), the intron of the Pp Luc+ pre-mRNA (siRNA-luc+ intron), or an unrelated gene, EGFP (siRNA-control). Livers were harvested 1 day after injection and homogenized in lysis buffer. Homogenates were assayed for Pp Luc+ and Rr Luc activity using the Dual Luciferase Assay Kit (Promega Corp.). The ratio of Pp Luc+ activity to Rr Luc activity in each sample was calculated and the ratios of all animals in each group was averaged. The average ratio in each group was scaled to the plasmids only group which was set at 100%. Error bars indicated 1 standard deviation. The data demonstrate that only the siRNA targeting the coding region of the Pp luciferase mRNA was effective in knockdown of Pp luciferase expression. Both the siRNA-luc+ an siRNA-luc+ intron siRNAs were equally able to anneal to the parent plasmid within the same transcription unit of the target gene. Nevertheless, only the siRNA with sequence identity to a region of the mature mRNA inhibited expression of the target gene. This result indicates that the co-delivered plasmid-encoded gene must be delivered to the cell and transcribed and have the transcript processed to mRNA before the siRNA becomes effective in knocking down expression through mRNA degradation.



Also note, in examples 1, 3 and 10 of the specification, that the control siRNA had identity to the ColE1replication origin, siRNA-ori. This sequence was present in the co-delivered plasmids. Thus, both the target gene specific siRNA and the control siRNA were equally able to interact with the plasmid DNA prior to delivery to the cell. However, only the target gene specific siRNA inhibited expression of the target gene.

The diagram below indicates the positions of the sequences identity between the expression plasmid and the siRNAs.